Characterization of a Microsomal Retinol Dehydrogenase: A Short-Chain Alcohol Dehydrogenase with Integral and Peripheral Membrane Forms That Interacts with Holo-CRBP (Type I)[†]

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ABSTRACT: Integral and peripheral forms of a microsomal retinol dehydrogenase (RoDH) have been distinguished in rat liver through differences in solubility, behavior toward affinity resins, and phase partitioning with Triton X-114. Despite physical differences, polyclonal antibodies raised against integral RoDH recognized peripheral RoDH. No obvious differences were observed in substrate specificity between the two forms. Integral and peripheral RoDH catalyzed retinal synthesis from *all-trans*-retinol bound to cellular retinol-binding protein, type I (CRBP), with similar K_m values of 0.6 and 0.4 μM, respectively. Both also discriminated against CRBP-bound *all-trans*-3,4-didehydroretinol and against 9-*cis*-retinol. Phenylarsine oxide inhibited both forms with IC_{50} values of 5 μM (integral) and 15 μM (peripheral). The more stable peripheral form has been reduced to two major polypeptides that migrate as 34 and 54 kDa bands on SDS−PAGE. The active site of this form has been associated with the 34 kDa polypeptide by covalent binding and inactivation with phenylarsine oxide and by cross-linking to holo-CRBP. Cross-linking required cofactor and was maximum with NADP, consistent with the ordered bisubstrate reaction mechanism of an NADP-supported dehydrogenase. The 34 kDa polypeptide has a subunit molecular weight and other attributes typical of short-chain alcohol dehydrogenases (SCAD) including the highly-conserved SCAD sequence WXLVNNAG, Zn^{2+} independence; inhibition by carbenoxolone (IC_{50} = 55 μM), and insensitivity to inhibition by ethanol and 4-methylpyrazole. Tight association between the 34 and 54 kDa polypeptides was demonstrated by their coelution through several columns and the precipitation of RoDH activity with either anti-34 kDa or anti-54 kDa antisera. Because SCAD normally occur as homomultimers, however, the 54 kDa polypeptide is not likely to be a subunit of the peripheral form. This work provides new evidence that the retinol-CRBP "cassette" serves as a substrate for a microsomal RoDH and further characterizes the RoDH.

Retinoic acid modulates gene expression during development as well as postnatally to control the differentiation state of numerous cell types in diverse organs (Lotan, 1988; Lohnes *et al.*, 1992; Reichel & Jacob, 1993). This suggests that the multi-loci biosynthesis of retinoic acid would be highly regulated (Napoli, *et al.*, 1991). Although it has been accepted for decades that two successive reactions convert retinol into retinoic acid, with retinal as an intermediate, progress in understanding regulation of retinoic acid biosynthesis has been hampered by a lack of detailed knowledge of the enzymes involved. One pathway recently elucidated relies on a microsomal, NADP-dependent RoDH that recognizes as substrate the "cassette" of retinol bound to CRBP[1] (Posch *et al.*, 1991; Napoli *et al.*, 1992). The retinal generated in microsomes from holo-CRBP subsequently supports cytosolic retinoic acid synthesis by a NAD-dependent retinal dehydrogenase; this retinal may be transferred from microsomes to cytosol by CRBP (Posch *et al.*, 1992). Besides serving as substrate in retinoic acid biosynthesis, holo-CRBP supports retinol esterification by LRAT (Ong *et al.*, 1988), whereas apo-CRBP inhibits LRAT (Herr & Ong, 1992) and stimulates retinyl ester hydrolysis (Ottonello *et al.*, 1987; Boerman & Napoli, 1991). This supports a model in which the ratio apo-CRBP/holo-CRBP determines the amount of retinol channelled into esters vs that converted into retinoic acid. As cellular retinol decreases, the increase in apo-CRBP would promote its own recharging by mobilizing retinyl esters while sparing holo-CRBP from retinol esterification, thus sustaining retinoic acid synthesis.

Since the discovery of CRBP, its function has been discussed but not resolved (Bashor *et al.*, 1973). The structure of CRBP consists of two perpendicular sets of five antiparallel β-strands. The resulting flattened "β-clam" structure enfolds retinol in a high-affinity binding pocket and isolates it from the cellular environment (Cowan *et al.*, 1993). The recently demonstrated roles for CRBP in retinoid metabolism provide concrete evidence of function but raise additional questions about the need for such a retinol binding protein in retinoid metabolism. This question seemingly has straightforward answers: CRBP would allow retinol access

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[1] Abbreviations: βME, β-mercaptoethanol; CRBP, cellular retinol-binding protein, type I; CRABP, cellular retinoic acid-binding protein; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; LRAT, lecithin−retinol acyl transferase; PAO, phenylarsine oxide; PBS, phosphate-buffered saline; RoDH, retinol dehydrogenase; SCAD, short-chain alcohol dehydrogenase; SASD, sulfosuccinimidyl-2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate; PAGE, polyacrylamide gel electrophoresis.

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only to enzymes capable of recognizing both binding protein and retinoid, imparting high specificity to retinol metabolism and preventing opportunistic retinoic acid synthesis. It would also protect retinol from undesired oxidation and cells from the membrane-disrupting potential of retinoids.

A role for holo-CRBP as a substrate in retinoid metabolism is consistent with related observations about its localization during embryogenesis. In the early mouse embryo, retinol concentrates in the regions that express CRBP (Gustafson *et al.*, 1993). For example, limb ectoderm expresses CRBP, whereas the underlying mesenchyma expresses CRABP type I (Gustafson *et al.*, 1993; Ruberte *et al.*, 1992). Ectoderm is most sensitive to vitamin A deficiency, indicating a requirement for retinoic acid, whereas mesenchyma is most sensitive to retinoic acid excess, indicating a protective role for CRABP. Thus, retinoic acid need, retinol, and CRBP expression colocalize, at least in this instance.

This work undertakes further characterization of the microsomal NADP-dependent RoDH and its interaction with holo-CRBP. One major goal was to identify the polypeptide(s) associated with RoDH activity. Through chromatography, chemical cross-linking with CRBP, covalent binding with and inhibition by PAO, and inhibition by carbenoxolone, a 34 kDa polypeptide, likely to be an SCAD, with two physically distinct forms (integral and peripheral membrane) has been associated with RoDH activity.

EXPERIMENTAL PROCEDURES

General. Retinoids were purchased from Eastman Kodak and were purified by HPLC (Napoli, 1986, Napoli, 1990). Activated Sepharose-4B, 2′,5′-ADP-Sepharose and Thiopropyl-Sepharose were purchased from Pharmacia-LKB. PAO-Sepharose-4B was synthesized as described (Berleth *et al.*, 1992). [^{125}I]PAO (1200 cpm/pmol), prepared as described (Hoffman & Lane, 1992), was a gift of Jun Li and Cecile Pickart, Department of Biochemistry, SUNY at Buffalo. Egg yolk L-α-phosphatidylcholine, Hunter's Titermax, pyridine nucleotide cofactors, detergents, and most other reagents were purchased from Sigma. Protein was determined by the dye-binding method with bovine serum albumin as standard (Bradford, 1976). Kinetic data were fitted with the microcomputer program Enzfitter (Leatherbarrow, 1987). Microsomes were prepared from the livers of male Sprague-Dawley rats (200 g, Harlan, Indianapolis, IN), as described previously (Napoli & Race, 1987), and were stored in 10 mM HEPES, 250 mM sucrose, 1 mM EDTA, and 2 mM DTT, pH 7.5 (50 mg of protein/mL) at −70 °C.

Preparation of CRBP. CRBP saturated with *all-trans*-retinol, which will be referred to as holo-CRBP, was prepared and purified from CRBP generated in *Escherichia coli* with the vector pMONCRBP (Levin *et al.*, 1988) as described previously (Posch *et al.*, 1991). Purified holo-CRBP had an A_{350}/A_{280} ratio of 1.4 and was stored in 20 mM HEPES, 150 mM KCl, 2 mM DTT, and 1 mM EDTA, pH 7.5 (100 nmol/mL) at −70 °C. CRBP-bound *all-trans*-3,4-didehydroretinol was prepared similarly. The identity of the ligand was verified by HPLC analysis of an aliquot of the Sephadex-G50 purified CRBP-bound 3,4-didehydroretinol. 9-*cis*-Retinol did not remain bound to CRBP during gel filtration, so the mixture of 9-*cis*-retinol and CRBP was not subjected to size-exclusion chromatography. Apo-CRBP was prepared and purified as was holo-CRBP except for saturation with retinol. The concentration of functional apo-CRBP was determined by saturating an aliquot with retinol, separating free and bound retinol by size-exclusion chromatography and determining the A_{350}/A_{280} ratio. The ratio A_{350}/A_{280} ratio was not affected in the presence of the phosphatidylcholine concentration used in the RoDH assays, indicating that the integrity of the holo-CRBP was not affected.

RoDH Assay. Assays were done in duplicate (duplicates were within 10% of their averages) at 37 °C for 30 min in 500 μL of 10 mM HEPES, 150 mM KCl, and 2 mM EDTA, pH 8, with 2 mM egg yolk L-α-phosphatidylcholine (added in 2 μL of ethanol) and 2 mM NADP. Partially purified peripheral and integral RoDH assayed in the absence of phosphatidylcholine had ~80% lower activity. Unless noted otherwise, the substrate was 2.5 μM holo-CRBP and 0.5 μM apo-CRBP to ensure complete binding of retinol and will be referred to as 2.5 μM holo-CRBP. [The final concentration of the detergent combination (see below) in the determination of K_m and substrate affinities for integral RoDH was no greater than 0.009%.] In one experiment, retinal reduction was assayed under similar conditions with retinal added in 2 μL of ethanol (1 μM final concentration) to a buffer containing 1.5 μM apo-CRBP and 100 μM NADPH in place of the holo-CRBP and NADP and will be referred to as 1 μM CRBP-retinal. Controls were assays done without cofactor. Reactions were quenched with 1 mL of 0.025 M KOH/ethanol and retinoids were extracted with 2.5 mL of hexane.

Assays of inhibition by carbenoxolone were done with 200 μg of microsomal protein in a total volume of 250 μL. The holo-CRBP/apo-CRBP, NADP, and other buffer constituents were the same as described above. Microsomes and inhibitor were preincubated for 10 min at 37 °C before starting the reaction by additions of substrate and cofactor.

HPLC Analysis. To quantify retinol and retinal, the hexane extracts of the assays were evaporated under a stream of nitrogen, and the residues were injected in 100 μL of hexane onto a normal-phase HPLC column (Dupont Zorbax-Sil Reliance cartridge column, 0.4 × 4 cm) (Napoli, 1986, 1991). A linear gradient was run from 4% tetrahydrofuran to 15% tetrahydrofuran in hexane for 5 min, followed by an additional 5 min of 4% tetrahydrofuran/hexane at 2 mL/min. Retinoids were detected with a Waters Model 484 tunable absorbance detector set for the first 5 min at 370 nm and at 325 nm for the remainder of the run and were quantified by comparing integrated peak areas to those of standards. The retention times for 13-*cis*-retinal, 9-*cis*-retinal, *all-trans*-retinal, 3,4-didehydroretinal, and *all-trans*-retinol were 1.35, 1.5, 2.0, 2.34, and 7.6 min, respectively.

Preparation of Peripheral RoDH. Routine preparation of peripheral RoDH involved gently stirring microsomes (10 mg of protein/mL) at 4 °C in a buffer of 20% glycerol and 20 mM potassium phosphate, pH 8 (buffer A). The mixture was allowed to stand for 10 min and was centrifuged at 100000*g* for 60 min. The supernatant was filtered through a 0.22 μm Gelman filter and stored at −20 °C. The pellet was rehomogenized in buffer A (~50 mg of protein/mL), stored at −70 °C, and used for some preparations of integral RoDH, as detailed below. Peripheral RoDH was also prepared by high salt (1 M NaCl) or high pH [pH 11 with 25 mM 3-(cyclohexylamino)propanesulfonic acid buffer in place of phosphate] microsomal extraction under conditions otherwise identical to those used for glycerol extraction.

A supernatant of a glycerol extract (2−5 mg of protein) was eluted through a PAO-Sepharose column (1 × 5 cm) in buffer A. The nonretained protein (1−4 mg), which accounted for all of the activity, was loaded in four portions onto a anion-exchange column (1 × 5 cm, Mono-Q, Pharmacia-LKB). RoDH activity was eluted with a 26 mL linear gradient from 0 to 650 mM NaCl in buffer A at a flow rate of 0.5 mL/min. Peak fractions of RoDH activity were pooled (~1.8 mL) and loaded onto a Sephadex-G200 column (1 × 36 cm) eluted at 0.2 mL/min with 2 mM phosphatidylcholine, 2 mM βME, and 150 mM KCl in buffer A.

Preparation of Integral RoDH. Integral RoDH was extracted from microsomes on ice for 1 h with 20 mM potassium phosphate, pH 8, 20% glycerol, 0.5 mM NADP and Triton X-100/Tween 80/Brij 92 (2/2/1, w/w/w) in a final concentration of 10 mg of protein and 25 mg of detergent combination per milliliter. The mixture was centrifuged at 100000*g* for 1 h. The supernatant (100 mg of protein) was applied to a PAO-Sepharose affinity column (1 × 5 cm) in buffer B (0.03% detergent combination, 20% glycerol, 2 mM βME, 0.06% ethanediol, and 2 mM phosphatidylcholine in 20 mM potassium phosphate buffer, pH 8.0). After being washed with 10 column volumes of buffer B, RoDH was eluted with a linear gradient of 0−25 mM DTT in 50 mL of buffer B. The DTT fraction was concentrated to 3 mL with an Amicon filtration unit (>10 kDa exclusion). One milliliter was applied to a Superose-12 column, and 2 mL was applied to a Sephadex G-200 column (1 × 36 cm); each was eluted with 150 mM KCl in buffer B.

Rabbit Antiintegral RoDH. Integral RoDH recovered from a PAO-affinity column was applied sequentially in 1 mL aliquots to a Superdex-200 column (1 × 36 cm) eluted at 0.2 mL/min with 150 mM KCl in buffer B. The protein that eluted in the void was subjected to 10% SDS−PAGE. The 34 and 54 kDa bands were identified by Coomassie blue staining, excised, and homogenized individually in 100 μL of distilled water by passing the mixtures through 21 gauge needles. Hunter's Titermax (1/1, v/v) was added to each antigen. Each preparation was injected subcutaneously into 10 spots (100 μL/per spot) on the backs of 2 kg female New Zealand white rabbits. Rabbits were exsanguinated after 6 months. A titer of 1/10 000 of each antiserum was visible by developing Western blots with alkaline phosphatase staining.

For the immunoprecipitation experiment, these antisera were purified by adjusting each to pH 8 by adding one-tenth volume of 1 M Tris-HCl, pH 8. The antisera were loaded individually onto peripheral RoDH affinity resins, equilibrated in 100 mM Tris-HCl, pH 8, containing leupeptine (50 μg/mL) and 2 mM phenylmethanesulfonyl fluoride. The mixtures were incubated overnight at 4 °C, and then the resins were washed sequentially with 10 column volumes each of 100 mM and 10 mM Tris-HCl, pH 8. Each IgG was eluted with 100 mM glycine, pH 3. Fractions (0.5 mL) were collected into 50 μL of 1 M Tris-HCl, pH 8. The concentration of IgG was determined spectrophotometrically (A_{280} of 1.0 = 0.75 mg of IgG/mL).

Synthesis of an RoDH Affinity Resin. A microsomal glycerol extract (~300 mg of protein) was eluted sequentially through a zinc-chelating column (3 × 4 cm) and a Thiopropyl-Sepharose column (2 × 4 cm) in buffer A, which removed ~75−83% of the protein but not RoDH activity and yielded a specific activity of ~95 pmol/(min·mg of protein). The recovered protein (50−75 mg) was loaded onto a 2′,5′-ADP Sepharose column (1 × 6 cm) and was washed with 10 column volumes of buffer A, followed by 5 mM NAD/NADH in buffer A. RoDH was eluted with 5 mM NADP in 15 mL of buffer A with a specific activity >500 pmol/(min·mg of protein). Seven hundred micrograms of protein from the NADP pool was added to 1 g of CH-Sepharose 4B (activated with 1 mM ice-cold HCl) in a total volume of 5 mL of buffer A with 2 mM βME. The mixture was incubated for 90 min at room temperature and overnight at 4 °C. Ninety percent of the protein was cross-linked as verified by SDS−PAGE of the supernatant. The resin was washed with 100 mM Tris-HCl in buffer A, pH 8, for 60 min before use, to block unreacted activated sites.

Phase Separation by Triton X-114. Triton X-114 was precondensed as described (Bordier, 1981). Microsomal membranes and the pellet from the glycerol extract of microsomes (10 mg of protein/mL) were solubilized with 2% Triton X-114 in 20 mM potassium phosphate, pH 8, at 4 °C. Insoluble material was removed by centrifugation at 100000*g* for 60 min at 4 °C. Each supernatant was incubated at 37 °C for 10 min and centrifuged at 5000*g* for 5 min. Triton X-114 was added to each supernatant to give a final concentration of 2%, and the procedure was repeated. The glycerol extract was also treated with 2% Triton X-114 and centrifuged twice at 100000*g* for 60 min at 4 °C.

Preparation of [^{125}I]SASD-Holo-CRBP. A glass reaction vial was coated with 1 mg of Iodogen (Pierce) dissolved in 100 μL of chloroform. Three milligrams of SASD (Pierce), a cleavable, heterobifunctional, cross-linking reagent, was dissolved in 50 μL of dimethyl sulfoxide in the dark. An aliquot was diluted 200-fold in PBS to make a working solution of SASD. A working solution of Na^{125}I was prepared by dissolving 1.2 μL of Na^{125}I (100 μCi/μL) in 30 μL of PBS. To the Iodogen-coated reaction vial was added 100 μL working solution of SASD and 10 μL working solution of Na^{125}I. After 30 s [^{125}I]SASD was added to 200 nmol of holo-CRBP in PBS and allowed to react in the dark for 60 min. The [^{125}I]SASD-holo-CRBP was separated from unreacted cross-linker on an Excellulose column (Pierce) with PBS. The specific activity was 8.5 × 10^4 cpm/nmol, and the concentration was 150 μM. CRBP retained its ligand, retinol, after these procedures, as determined by the ratio A_{350}/A_{280}.

SDS−PAGE, Autoradiographs, and Immunoblotting. Ten percent SDS−PAGE in the presence of βME was performed as described (Laemmli, 1970), with the Hoefer Scientific Mighty Small slab gel electrophoresis unit. Gels were either fixed overnight in 50% methanol and silver stained as described (Wray *et al.*, 1981), dried onto 3 mm Whatman paper, and autoradiographed with an intensifying screen at −70 °C, or transferred to a PVDF membrane (Bio-Rad) for immunoblotting. Autoradiographs were quantified by densitometry. Transfers were effected from SDS−PAGE gels to PVDF membranes with the Bio-Rad miniblotting apparatus. After incubating the PVDF membrane for 30 min at room temperature in 3% dried milk powder and 0.1% Tween-20 in PBS, primary antibody was added to the membranes and the resulting solution was incubated overnight at 4 °C. The membranes were developed by the Bio-Rad alkaline phosphatase procedure.

Amino Acid Sequence Analysis. Peripheral RoDH, isolated as described in Experimental Procedures in *Preparation of Peripheral RoDH* through the Sephadex G200 column step, and integral membrane RoDH, partially purified through the Superdex step as described for antibody production, were subjected individually to 10% SDS−PAGE (1.5 mm) and transferred (70 V, 60 min) to a nitrocellulose membrane in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid buffer, pH 11, with 10% methanol. The 34 kDa band was identified by staining with 0.2% Ponceau S in 1% acetic acid and excised. The membrane was destained with 1% acetic acid and rinsed in distilled water. The N-terminal amino acid sequence was determined on the peripheral RoDH sample and part of the integral RoDH sample by the SUNY Buffalo Protein Chemistry Facility run by Dr. Ed Niles. The remaining integral RoDH was digested *in situ* with trypsin by Dr. William Lane of the Harvard Microchemistry Facility, Harvard University, MA. Four peptides were isolated by HPLC and sequenced by Edman degradation.

RESULTS

Integral RoDH. The initial approach to identifying the RoDH polypeptide relied on detergent solubilization of microsomes with a combination of three nonionic detergents, Triton X-100, Tween 80, and Brij 96. A 2.5-fold excess of this combination relative to microsomal protein (mg/mg) solubilized 40−60% of the microsomal protein and stimulated RoDH activity 8-fold for a short time: activity decreased markedly during 5 h at 4 °C.

Because PAO inhibits microsomal RoDH activity (see below), PAO affinity chromatography was applied to the isolation of the detergent-extracted RoDH. The PAO-Sepharose resin reproducibly partitioned the activity into two fractions: ~30−60% of the activity and 2−10% of the protein was retained, depending on the experiment. The retained RoDH was eluted with a buffer containing DTT (Figure 1). The RoDH recovered in the DTT fraction (subsequently identified as integral RoDH) was stable at −20 °C for about 3 weeks but was unstable after any other purification procedure. Therefore, kinetic studies on integral RoDH were done with the DTT fraction eluted from the PAO column.

The integral RoDH eluted from the PAO column with DTT was divided into two portions: each was analyzed by size-exclusion chromatography, but each lost activity within 4 h after recovery (Figure 2). Nevertheless, the activity from the G200 column was correlated with the major bands at 34 and 54 kDa, although the possibility could not be excluded from these data alone that a minor band, e.g., the one at 45 kDa, might represent RoDH activity. A comparison of the peak activity fractions of the G200 column (fractions 12−15) with the peak activity fractions of the Superose-12 column (fractions 7 and 8) excluded the 45 kDa band and several of the other minor bands observed in the G200 fractions. Evaluation of the two columns together suggested that the 34 and 54 kDa bands were likely candidates for RoDH. Therefore, antibodies were raised against these two polypeptides.

Peripheral RoDH. In an attempt to find conditions that might preserve RoDH activity, a second approach used detergent-free extraction of microsomes with a buffer containing 20% glycerol. This removed 20−30% of the protein and ~20% of RoDH units from the microsomes. This form of RoDH, extracted with the peripheral membrane proteins (peripheral RoDH), was stable when frozen at −20 or −70 °C for 2−3 months but tended to be unstable after further purification. Two other methods of removing peripheral proteins from membranes, high salt (1 M NaCl) and high pH (pH 11) extraction (Fairbanks *et al.*, 1971; Steck & Yu, 1973; Tanner & Gray, 1971), also removed ~20% of RoDH activity and 20−30% of the protein from the microsomes. RoDH extracted with high salt or high pH, however, was not stable after freezing and thawing.

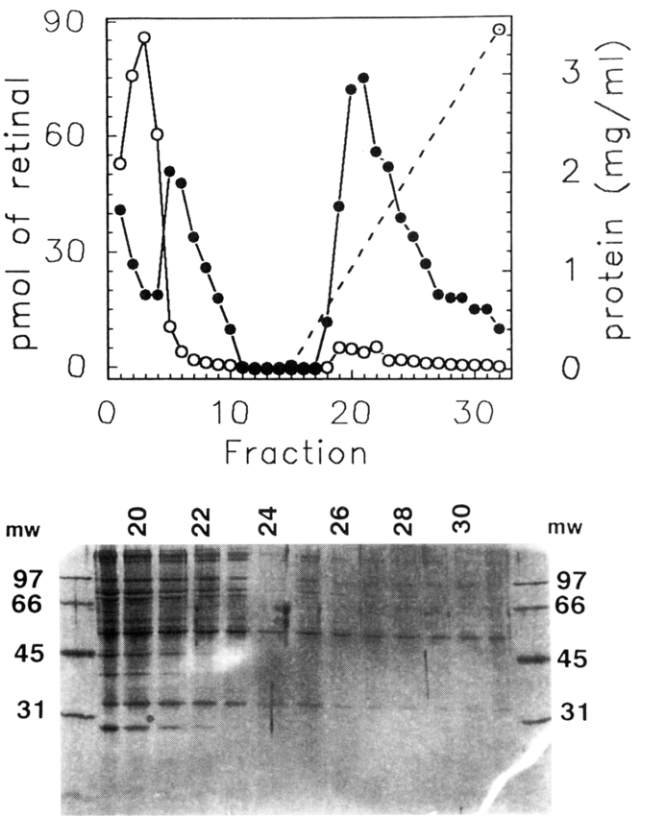

FIGURE 1: PAO-affinity chromatography of solubilized microsomes. (Top) Solubilized microsomes were eluted from a PAO-affinity column at 0.5 mL/min, and 2 mL fractions were collected. Protein (open circles) and RoDH activity with 2.5 μM holo-CRBP (0.2 mL aliquots) were assayed (filled circles). The dashed line indicates the gradient from 0 to 17 mM DTT. (Bottom) An SDS−PAGE gel was run on 20 μL aliquots from fractions 19 through 31 and silver-stained (lanes 2−14; fractions are noted at the tops of alternate lanes). Lanes 1 and 15 show molecular weight markers.

The best results for isolating peripheral RoDH were achieved with the following small-scale method, which minimized the time of handling. A glycerol extract of microsomes (~4 mg of protein) was applied to a PAO-affinity column. *All of the activity eluted in the flow through.* This peripheral RoDH [specific activity of 49 pmol/(min·mg of protein)] was then eluted from an anion-exchange column in a NaCl gradient (Figures 3 and 4). The most active fractions, 20 and 21, were applied to Sephadex-G200. To conserve the limited quantity of enzyme obtained by this small scale preparation, the amount of protein recovered was not quantified. Sephadex-G200 yielded two major bands of 54 and 34 kDa that migrated with activity and removed many of the minor bands observed after the anion-exchange step (Figure 5). The specific activity of RoDH recovered from the Sephadex column could not be determined precisely,

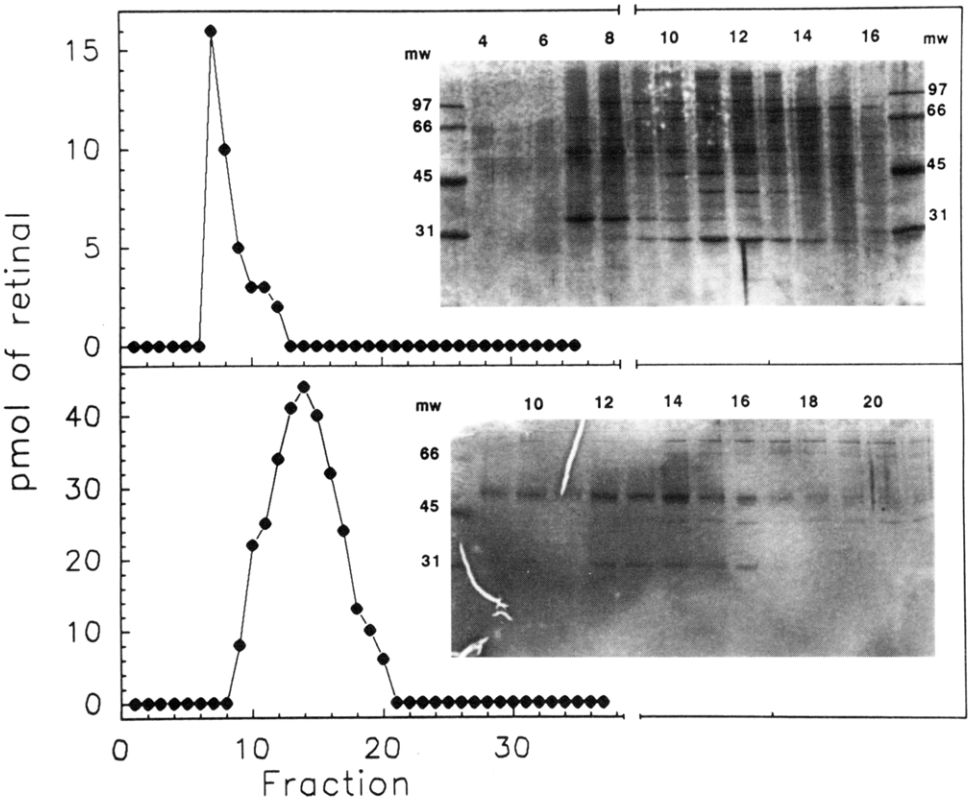

FIGURE 2: Size-exclusion chromatography of integral RoDH. Aliquots of RoDH recovered from the DTT-gradient of the PAO column (Figure 1) were applied to a Superose-12 column and eluted at a flow rate of 0.5 mL/min (top) or to a Sephadex G200 column and eluted at a flow rate of 0.2 mL/min (bottom). In each case 0.5 mL fractions were collected, and 0.35 mL aliquots were assayed for RoDH activity with 2.5 μM holo-CRBP. The inserts show silver-stained SDS−PAGE gels of the designated fractions.

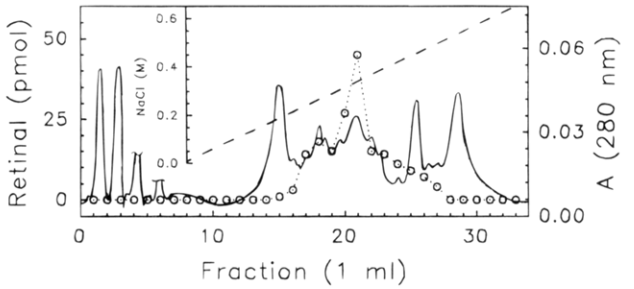

FIGURE 3: Anion-exchange chromatography of peripheral RoDH. A glycerol extract of microsomes (peripheral RoDH), recovered from the wash-through of PAO-Sepharose, was applied to an anion-exchange column in four portions as described in Experimental Procedures. After the last portion was applied, a linear gradient was run from 0 to 0.65 M NaCl (dashed line). Aliquots (0.1 mL) of the fractions were assayed for RoDH activity with 2.5 μM holo-CRBP (open circles). Protein was monitored at 280 nM (solid line).

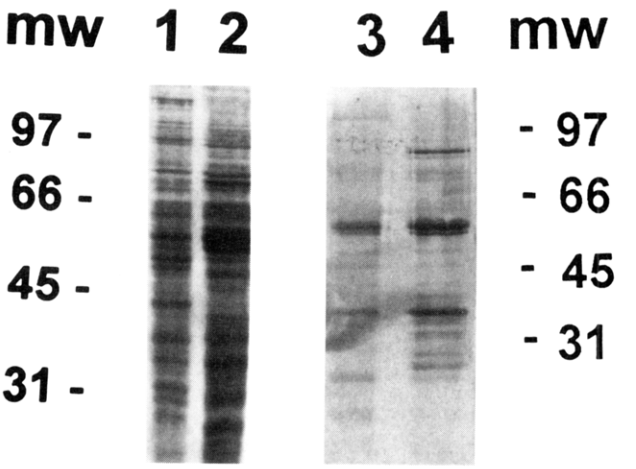

FIGURE 4: SDS−PAGE analysis of peripheral RoDH after anion-exchange chromatography. Ten percent gels were run and silver stained as described in Experimental Procedures. (Lane 1) Protein remaining in the microsomal pellet after glycerol extraction; (lane 2) protein in the supernatant of a glycerol extract; (lanes 3 and 4) protein in fractions 20 and 21 recovered from an anion-exchange column (see Figure 3). Migrations positions of molecular weight markers for lanes 1 and 2 are indicated on the left and on the right for lanes 3 and 4.

because the protein was too low to measure by conventional techniques. By estimating the amount of protein from the intensity of the gel band, the specific activity was estimated as ~500 pmol/(min·mg of protein). This sample remained active only ~2 h.

Two forms of RoDH Are Distinguished by Affinity Resins. Microsomes were extracted with the detergent-free glycerol buffer to remove peripheral RoDH. The resulting pellet then was extracted with the combination of detergents described in Experimental Procedures under *Rabbit Antiintegral RoDH* to solubilize integral RoDH. Integral RoDH was retained totally by PAO-Sepharose and Thiopropyl-Sepharose, and was eluted in each case with DTT, but was *not* retained by 2′,5′-ADP-Sepharose (data not shown). In contrast, the peripheral RoDH was not retained by the PAO-Sepharose nor the Thiopropyl-Sepharose affinity columns. It was, however, retained by 2′5′-ADP-Sepharose and was eluted with NADP (see Experimental Procedures under *Synthesis of an RoDH Affinity Resin*). Thus, integral RoDH behaved differently than peripheral RoDH with three affinity resins.

Two RoDH forms Are Distinguished by Phase Partitioning. Phase separation of microsomes with Triton X-114 resulted in RoDH in both the detergent and aqueous phases, deter-

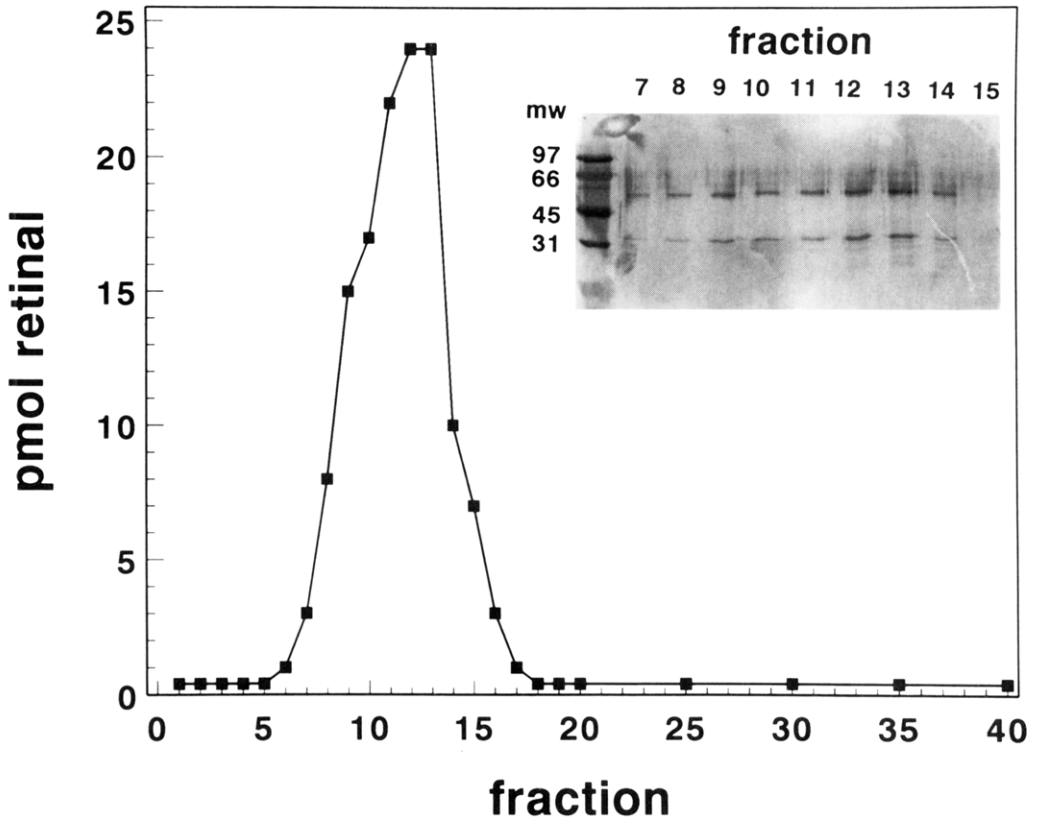

FIGURE 5: Size-exclusion chromatography of peripheral RoDH. Peripheral RoDH recovered from the pool of fractions 20 and 21 of the anion-exchange column described in Figure 3 was applied to a Sephadex-G200 column as described in Experimental Procedures. Fractions (0.5 mL each) were collected, and aliquots (0.35 mL) were assayed for RoDH activity with 2.5 μM holo-CRBP. The insert shows a silver-stained SDS−PAGE gel of 20 μL of the designated fractions. The bands at 60 and 67 kDa are artifacts caused by βME in the sample buffer (30).

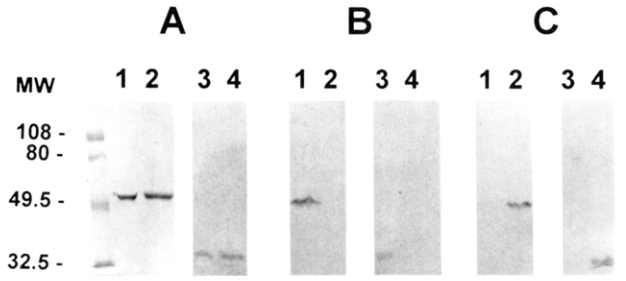

FIGURE 6: **6.** Phase separation of microsomal, peripheral and integral RoDH. Phase partition with Triton X-114, as described in Experimental Procedures, was performed on (A) Microsomes, (B) the supernatant of a glycerol extract of microsomes (peripheral RoDH), (C) the pellet from a glycerol-extract of microsomes (integral RoDH). The aqueous phases (1, 3) and the detergent phases (2, 4) were subjected to SDS−PAGE and were analyzed by immunoblotting using anti-integral 54 kDa (1, 2) or anti-integral 34 kDa (3, 4) antiserum at dilutions of 1/5000 and 1/2500, respectively.

mined by Western blotting of each phase with antibodies raised against the 34 and 54 kDa polypeptides from integral RoDH (Figure 6A, lanes 1−4). In contrast, RoDH in the glycerol extract was observed only in the aqueous layer, consistent with a peripheral membrane protein (Figure 6B, lanes 1 and 3). RoDH in the pellet of the glycerol-extracted microsomes localized completely to the detergent layer, consistent with an integral membrane protein (Figure 6C, lanes 2 and 4).

Kinetic Properties of RoDHs. The two forms of RoDH were compared to determine whether they differed markedly in kinetic properties or substrate specificity. Peripheral RoDH activity in the glycerol extract had an apparent K_m for holo-CRBP of 0.4 ± 0.1 μM (mean ± SE of four experiments, each with nine concentrations of substrate between 0.05 and 10 μM) and a V_{max} of 59 ± 10 pmol of retinal/(min·mg). Integral RoDH, prepared from detergent-solubilized microsomes by DTT elution of a PAO-affinity column, had an apparent K_m for retinal synthesis from holo-CRBP of 0.6 μM and a V_{max} of 115 pmol/(min·mg) (mean of two experiments, each with nine concentrations of substrate between 0.05 and 10 μM). Because the K_m values compare well with our previously reported K_m for holo-CRBP and RoDH of 1.6 μM, obtained with rat liver microsomes (Posch *et al.*, 1991), the milieu, microsomal lipids vs phosphatidylcholine, apparently had little effect on RoDH affinity for holo-CRBP.

Both forms of RoDH had similar relative activities toward a variety of substrates (Table 1). Both accepted free and CRBP-bound retinol and free 3,4-didehydroretinol as comparable substrates. The rate of 3,4-didehydroretinal formation from CRBP-bound 3,4-didehydroretinol with both forms, however, was at least 10-fold less than that from free 3,4-didehydroretinol. 9-*cis*-Retinol in the presence or absence of CRBP was not a competent substrate for either form of RoDH. 13-*cis*-Retinol, free or in the presence of CRBP, was not recognized efficiently as substrate by peripheral RoDH.

Sensitivity of RoDH to Inhibitors. The IC_{50} values for PAO inhibition of microsomal and integral RoDH were 2 and 5 μM, respectively; the IC_{50} for inhibition of peripheral RoDH was 15 μM (Figure 7). The last value is surprising

Table 1: Substrate Specificities of Integral and Peripheral Membrane RoDH[a]

retinoid (5 μM)	CRBP (7 μM)	RoDH relative activity[b] integral	peripheral
all-trans-retinol	−	100	100
	+	66	70
3,4-didehydroretinol	−	116	74
	+	11	ND
9-*cis*-retinol	−	ND[c]	ND
	+	ND	ND
13-*cis*-retinol	−	—[d]	ND
	+	—	ND

[a] Assays were done as described in Experimental Procedures with 25 μg of protein eluted from the PAO-Sepharose column with DTT (integral RoDH) or with 70 μg of protein from a glycerol extract of microsomes (peripheral RoDH). [b] Activity relative to rates of retinal formation of 180 and 42 pmol/(min·mg of protein) for integral and peripheral RoDH, respectively. [c] ND, not detected, less than 2 pmol of retinal formed. [d] —, not done.

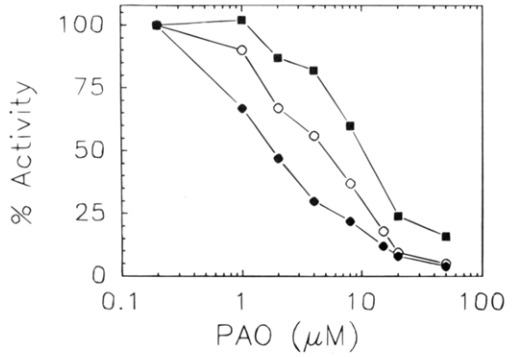

FIGURE 7: PAO Inhibition of RoDH. Activity was assayed with 2.5 μM holo-CRBP and 2 mM NADP as described in Experimental Procedures. Samples were preincubated with the indicated concentrations of PAO for 10 min at 37 °C in the presence of cofactor: microsomal RoDH, 150 μg of microsomal protein (filled circles); integral RoDH, 150 μg of protein left in the microsomal pellet after glycerol extraction (open circles); peripheral RoDH, 70 μg of glycerol extract supernatant protein (filled squares).

in view of the failure of the peripheral form to bind to PAO-Sepharose.

Carbenoxolone (1 mM), an inhibitor of SCAD, inhibited RoDH activity in microsomes by 95−100%. The IC_{50} for carbenoxolone inhibition of microsomal RoDH activity, determined with holo-CRBP as substrate, was 55 ± 32 μM (mean ± SE of three experiments, each with seven to nine concentrations of carbenoxolone between 5 and 1000 μM).

Covalent Binding of [^{125}I]PAO to the 34 kDa Polypeptide. To identify the polypeptide(s) that contained the site of PAO binding, [^{125}I]PAO was incubated with RoDH partially purified by two different strategies. The mixtures were analyzed by SDS−PAGE in the absence of thiol reducing agents. Peripheral RoDH, obtained from an anion-exchange column as depicted in Figure 3, yielded an intense band at ~82 kDa after [^{125}I]PAO-labeling when it was applied to SDS−PAGE without prior thermal denaturation. Thermal denaturation yielded several bands, with the most intense at 34 kDa; consistent with native RoDH occurring as a heterodimer of 34 and 54 kDa polypeptides (Figure 8). Peripheral RoDH enriched by a different strategy, which included elution from a 2′,5′-ADP affinity column with NADP (see Experimental Procedures under *Synthesis of an RoDH Affinity Resin*), yielded only a single intense band at 34 kDa after thermal denaturation, despite the presence of

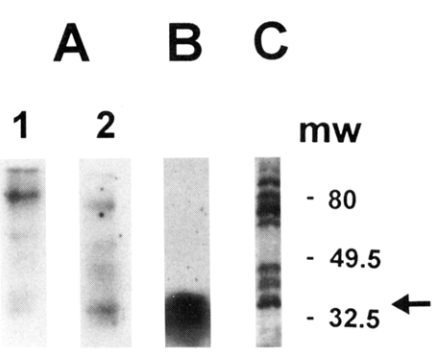

FIGURE 8: [^{125}I]PAO labeling of peripheral RoDH. For these experiments, peripheral RoDH from a microsomal glycerol extract was enriched as described in Experimental Procedures under *Preparation of peripheral RoDH* up to and including the anion-exchange step or under *Synthesis of an RoDH Affinity Resin* up to and including the 2′,5′-ADP affinity step. In both cases, ~20 μg of protein was incubated with 200 pmol of [^{125}I]PAO for 30 min at 4 °C in 0.1 mL of 20% glycerol in 20 mM potassium phosphate, pH 8.0. Samples were diluted 2:1 with sample buffer without reducing agents (except C) and analyzed by SDS−PAGE: (A) autoradiograph of the reaction of RoDH from the anion-exchange column; (B) autoradiograph of the reaction of RoDH from the NADP fraction of 2′,5′-ADP Sepharose; (C) silver-stained protein recovered from the NADP fraction of 2′,5′-ADP Sepharose. The samples were applied to the gel without boiling (A1) or after boiling (A2, B, and C). The position of the 34 kDa band is denoted by the arrow. The protein composition of the RoDH from the anion-exchange column used in *A* is shown in Figure 4 (lanes 3 and 4).

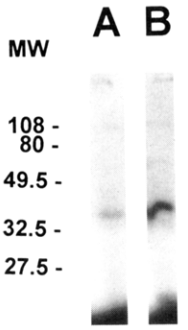

FIGURE 9: Cross-linking between RoDH and [^{125}I]SASD-CRBP. Microsomes (A) or a glycerol extract of microsomes (B) 200 μg of protein, were allowed to react with 19 μM [^{125}I]SASD-holo-CRBP and 5 mM NADP in 200 μL of RoDH assay buffer. After a 15 min incubation at 37 °C in the dark, the samples were irradiated at 360 nm with a model UVSL-25 Mineralight for 20 min. The cross-linker was inactivated by boiling for 2 min in the presence of βME (20 μL). Samples were analyzed by autoradiography of SDS−PAGE gels.

multiple polypeptides in the source of RoDH used in the experiment (Figure 8). Notably, [^{125}I]PAO binding was observed only with enzymatically active RoDH.

Cross-Linking with Holo-CRBP. To identify the site of CRBP interaction, CRBP was covalently labeled with a UV-activated, cleavable cross-linking reagent and was allowed to react with microsomes or a glycerol extract of microsomes. The reagent was radioiodinated such that the iodine would be transferred covalently from the CRBP to any target protein upon activation and cleavage. A dominant radiolabeled band was observed consistently at ~37 kDa upon allowing [^{125}I]-SASD-holo-CRBP to react with microsomes or the peripheral extract of microsomes in the presence of NADP, despite the complex protein composition of the sources of RoDH (Figure 9). In the absence of cofactor this band was not observed, and its intensity in the presence of NADPH or NAD was 31% and 25% of that observed with NADP, respectively

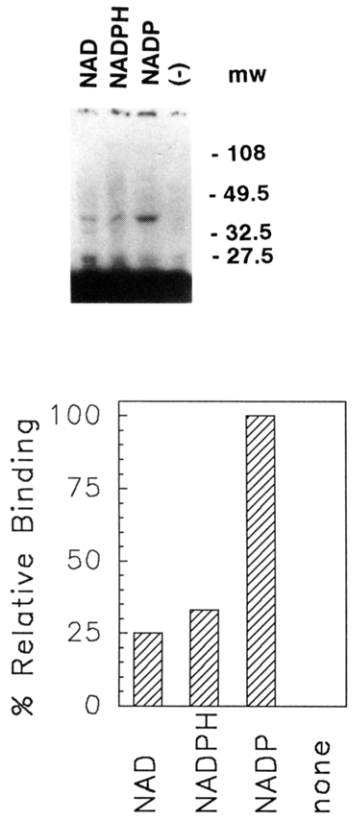

FIGURE 10: Cofactor requirement for cross-linking of CRBP. Either 3.8 μM (expt 1) or 11.3 μM [^{125}I]SASD-holo-CRBP (expt 2) were used with 250 μg of protein from a microsomal glycerol extract. Cross-linking by UV irradiation for 20 min was done after dark incubation for 15 min at 37 °C with no cofactor or 5 mM of NADP, NADPH or NAD. (Top) Autoradiograph of SDS−PAGE analysis of experiment 1. (Bottom) Data are plotted as the averages of the two experiments (the results of each were within 5% of their means) normalized to cross-linking in the presence of NADP. The bands at the top of the figure were produced by aggregated polypeptides in the stacking gel.

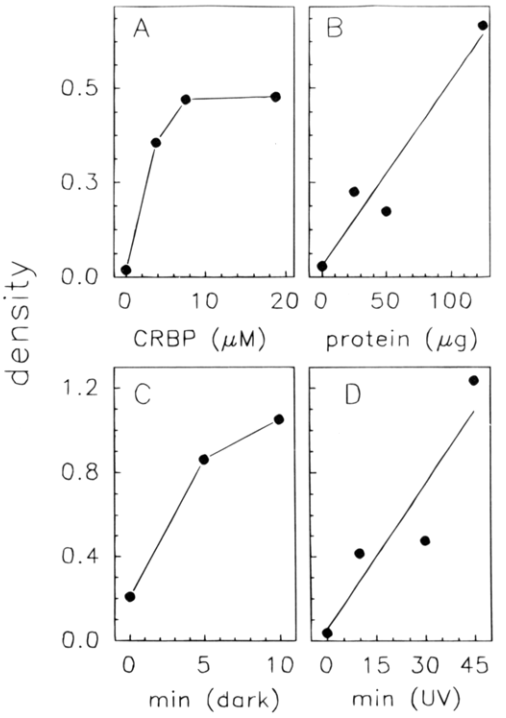

FIGURE 11: Effects of time, protein, and CRBP concentrations on cross-linking with CRBP. The degree of cross-linking was determined by densitometry of autoradiographs obtained from SDS−PAGE analyses. Standard conditions used 250 μg of protein from a glycerol extract of microsomes, 37.5 μM [^{125}I]SASD-holo-CRBP, and 2 mM NADP with dark incubation for 15 min, followed by UV irradiation for 20 min. Varied were (A) [^{125}I]SASD-holo-CRBP concentrations, (B) glycerol extract protein, (C) dark incubation time, and (D) photolysis time.

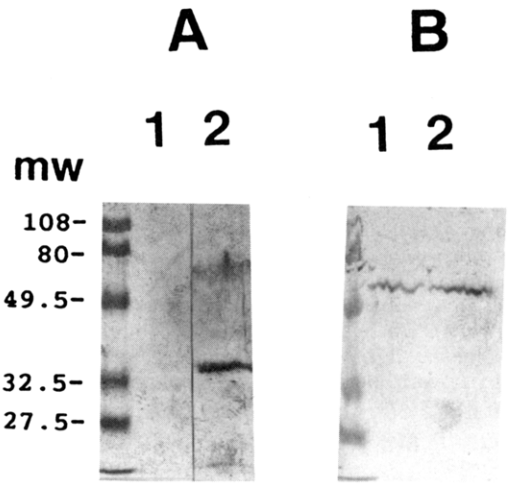

FIGURE 12: Immunochemical analysis of cross-linked RoDH. A microsomal glycerol extract was subjected to the cross-linking protocol described in the legend of Figure 9 and irradiated in the presence, (lane 1) or absence (lane 2) of [^{125}I]SASD-holo-CRBP. Samples were analyzed by SDS−PAGE and immunoblotted with: (A) anti-integral 34 kDa antiserum, 1/2000 dilution and (B) anti-integral 54 kDa antiserum, 1/5000 dilution.

(average of two experiments) (Figure 10). In some experiments, another conspicuous band at 25 kDa was observed, but its intensity was not affected by the presence or absence of NADP, nor was it always observed. As determined with peripheral RoDH from a glycerol extract, the intensity of the 37 kDa band was linearly dependent on protein to ~100 μg and on the duration of UV irradiation for at least 45 min. The extent of cross-linking also increased with the time of preincubation in the dark of the complete cross-linking mixture up to at least 10 min and was maximum at 7.5 μM holo-CRBP (Figure 11).

Antiintegral 34 kDa antibodies were used in Western blot analysis of the radiolabeled band. No 34 kDa band was observed by immunoblotting whenever cross-linking occurred, nor was the radioiodinated band detected by the anti-34 kDa antibodies (Figure 12). In contrast, immunodetection of the 54 kDa polypeptide was unaffected by the cross-linking protocol.

Association of the 34 and 54 kDa Bands. Each of the antisera raised against the 34 and 54 kDa polypeptides from the integral RoDH removed peripheral RoDH activity from a glycerol extract of microsomes, as might be anticipated if the two proteins represented by these bands were associated (Figure 13). Note that this result also demonstrates that the anti-34 kDa antiserum raised against *integral* RoDH immunoprecipitated *peripheral* RoDH because peripheral RoDH was the sole source of the RoDH enzymatic activity used in the experiment.

Amino Acid Sequence of the 34 kDa Band. The N-terminal amino acid sequences of integral and peripheral RoDH were identical: MWLYLLALVG. In addition, four internal polypeptides were separated by HPLC from a trypsin digest of integral RoDH and were sequenced. Three of these, YSPGWDAK, VAIIEPGGFK, and ELTYFGVK, had no

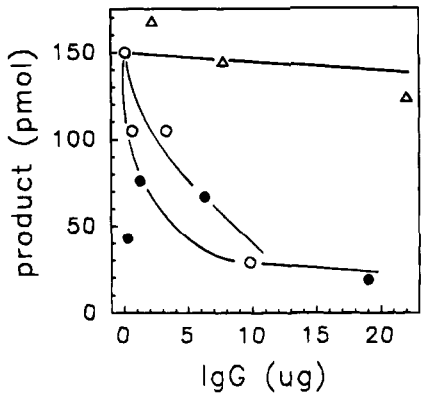

FIGURE 13: Immunoprecipitation of peripheral RoDH activity. A glycerol extract of microsomes (50 μg of protein) was incubated with antigen-Sepharose purified anti-integral 54 kDa (filled circles) or anti-integral 34 kDa (open circles) antisera overnight at 4 °C in 0.1 mL of 20% glycerol in 20 mM potassium phosphate, pH 8.0. Incubates were treated with 50 μL of protein A Sepharose in PBS for 2 h at 4 °C, and supernatants were prepared by centrifugation (14000*g* for 2 min). RoDH activity was assayed by monitoring reduction of 1 μM CRBP-retinal. Control supernatants were generated with IgG obtained from protein A purified preimmune serum (triangles).

significant homology to proteins in the Swiss protein data base. One, however, LWGLVNNAGISVPV-PNE-M, contained a sequence, WXLVNNAG, well conserved among SCAD (Persson, 1991).

DISCUSSION

A major goal of this work was to identify the catalytic polypeptide of RoDH for further characterization, raising antibodies, and cloning. Total purification was not achieved because the RoDH lost activity as purity increased, a common occurrence with membrane-associated enzymes. Nevertheless, two forms of RoDH were distinguished, their comparative substrate specificities and sensitivities to inhibitors were determined, physical interaction with holo-CRBP was demonstrated, and the catalytic polypeptide was identified.

Three different observations indicated that rat liver microsomal RoDH occurs in at least two physically distinct forms. First, a peripheral RoDH was solubilized by each of three methods (high salt, high pH, and glycerol extraction) commonly used to liberate peripheral proteins from membranes. Second, an integral RoDH was solubilized only with detergent. Third, the two forms were separated completely by Triton X-114 phase partitioning. Triton X-114 and aqueous buffers are miscible at 4 °C, but above 20 °C they separate into two discrete phases, generally relocating the hydrophobic proteins with the detergent phase and the more hydrophilic proteins in the aqueous phase (Bordier, 1981). The two forms were also distinguished by their contrasting affinity for functional group and pyridine nucleotide resins. The occurrence of two physically distinct forms suggests differences in structure. These could be in amino acid sequence (albeit not at the N-termini) or in posttranslational modifications; either could affect external topography. There were no discernible differences in substrate specificity, however, and relatively modest differences in sensitivity to PAO inhibition. The two forms were not distinguishable immunochemically, at least with the antibodies available presently. The physiological significance of distinct forms of RoDH remains unknown; however, the two forms could be distributed in different cell types and/or be regulated differently; one might be a precursor of the other. Future work will address these issues and whether the two forms are isozymes or are generated posttranslationally.

The next enzyme in the pathway of retinoic acid synthesis, a rat liver cytosolic NAD-dependent retinal dehydrogenase (Posch *et al.*, 1992), recognizes *all-trans*- and 9-*cis*-retinoids nearly equally well ($V_m/K_{0.5}$ of 2.0 and 1.5, respectively) but discriminates against 13-*cis*-retinal (El Akawi & Napoli, 1994). The broader substrate specificity of the retinal dehydrogenase would allow it to synthesize retinoic acids from both *all-trans*-retinal and 9-*cis*-retinal, available from dietary 9-*cis*-retinol, metabolism of 9-*cis*-β-carotene (Stahl *et al.*, 1993), or isomerization of *all-trans*-retinoids (Rando, 1994). In contrast, both forms of microsomal RoDH recognize *all-trans*-retinoids specifically and discriminate against *cis*-isomers. Any 9-*cis*-retinol dehydrogenase activity would have to be a different enzyme.

Recognition of 3,4-didehydroretinol as substrate for RoDH in the free but not the CRBP-bound state, in contrast to the results with free and bound *all-trans*-retinol, shows that not all CRBP−retinoid complexes can serve as efficient substrates for metabolism of their ligands. A similar phenomenon has been observed with CRABP(I) (Fiorella & Napoli, 1994). *all-trans*-Retinoic acid bound to CRABP has a turnover half-time in rat testes microsomes similar to that for free retinoic acid (35 vs 40 min, respectively). Free-4-oxoretinoic acid in the same experiment had a half-time of 9 min. Upon binding to CRABP, 4-oxoretinoic acid was not metabolized. These observations suggest retinoids may have their metabolism altered by the nature of their interactions with retinoid binding proteins. This may be important in determining which activated retinoids exert influence in specific loci. For example, in the adult human, conversion of retinol into 3,4-didehydroretinol and the latter into 3,4-didehydroretinoic acid occurs in keratinocytes (Randolph & Simon, 1993), which express only low levels of CRBP (Siegenthaler *et al.*, 1990).

Anion-exchange, size-exclusion, or affinity chromatography of peripheral RoDH yielded a sample consisting of two major electrophoretic bands (~34 and ~54 kDa) which comigrated with the enzymatic activity. The combination of radioiodinated PAO, a potent inhibitor of RoDH activity which forms covalent adducts between spatially proximal sulfhydryl groups (Hoffman & Lane, 1992; Jauhiainen, 1988), and chemical cross-linking with holo-CRBP, a substrate for RoDH, associated the 34 kDa polypeptide with the catalytic activity. The 34 kDa polypeptide was the only one recognized by both of these reagents and was the most intensely bound polypeptide with each.

The dependence on cofactor of RoDH cross-linking with holo-CRBP, with maximum cross-linking in the presence of NADP, showed that holo-CRBP delivered its radiolabeled cross-linker to an enzyme that followed an ordered bisubstrate reaction mechanism, i.e., an NADP-dependent dehydrogenase (Dalziel, 1963; Wratten & Cleland, 1963; Eklund *et al.*, 1981). Because microsomes and the glycerol extract contain numerous proteins, the specificity of CRBP for the 34 kDa polypeptide was demonstrated by its predominant labeling in these highly heterogeneous sources of RoDH. CRBP recognizes microsomal activities other than RoDH as substrate and/or effector, however, including LRAT (Ong

et al., 1988; Herr & Ong, 1992), cholate-independent retinyl ester hydrolase(s) (Boerman & Napoli, 1991), and possibly others. The relatively faint bands could represent these activities. For example, recently Shi *et al.* (1993) have associated a 25 kDa polypeptide with the catalytic site of LRAT.

Modification of RoDH with the cross-linker resulted in slower migration on SDS−PAGE and loss of recognition by the anti-34 kDa antiserum. Although the antiserum was polyclonal, possibly the antigenic determinant was contributed by limited epitopes that were altered by cross-linking. Alternatively, the amount of the modified enzyme that entered the gel might have been too small to detect by Western blot analysis. Cross-linking of membrane proteins results commonly in substantial aggregation.

Amino acid sequencing of peptides generated by trypsin digestion of the 34 kDa band revealed a sequence (WGLVNNAG) highly conserved in the superfamily of SCAD (Persson *et al.*, 1991). About 22 SCAD have been characterized (Persson *et al.*, 1991; Marks *et al.*, 1992; DeLong *et al.*, 1993). These dehydrogenases have in common a subunit size between 28 and 35 kDa. None contain Zn^{2+}. They are distinct from the family of medium-chain (classical) alcohol dehydrogenases associated with ethanol metabolism, which have subunits of ~40 kDa and rely on Zn^{2+} as a prosthetic group. Carbenoxolone, the steroidal aglycone of the licorice-derived compound glycyrrhizin, inhibits SCAD such as rat kidney and liver 11β-hydroxysteroid dehydrogenase (Monder *et al.*, 1989; Baker & Fanestil, 1991; Edwards *et al.*, 1993), rat liver 3α-hydroxysteroid dehydrogenase (Akao *et al.*, 1992), *Streptomyces hydrogenans* 3α,20β-hydroxysteroid dehydrogenase (Ghosh *et al.*, 1992), and 15-hydroxyprostaglandin dehydrogenase (Muramatsu *et al.*, 1984; Baker & Fanestil, 1991) with K_i values in the micromolar range. The characteristics of RoDH are similar to those of SCAD and are distinct from those of medium-chain alcohol dehydrogenases. RoDH has a subunit molecular weight similar to that of SCAD and is inhibited by carbenoxolone with a μM IC_{50}; also, it does not appear to be Zn^{2+} dependent because it is not inhibited by either 8 mM 1,10-phenanthroline or 50 mM EDTA.[2] Moreover, 100 mM 4-methylpyrazole, an inhibitor of human class I and II alcohol dehydrogenases and rat class 1 and 3 alcohol dehydrogenases (Vallee & Bazzone, 1983; Julia *et al.*, 1987), and 860 mM ethanol do not inhibit RoDH (Posch *et al.*, 1991).

We have obtained a cDNA clone of the entire coding region of the 34 kDa band. Degenerate primers, designed from the amino acid sequence of two of the peptides generated by the trypsin digest, were used in conjunction with reverse-transcriptase polymerase chain reaction with rat liver mRNA as the initial template (Chai *et al.*, 1995). This clone encodes all five of the polypeptides sequenced (four internal and the N-terminus), including the one containing the SCAD-characteristic WXLVNNAG sequence. This clone also contains the sequence YCISK 60 amino acids C-terminal to WGLVNNAG. SCAD are characterized by the highly conserved sequence YXXSK and this positioning relative to the WGLVNNAGI sequence. The tyrosine and lysine are considered essential active site residues (Persson *et al.*, 1991; Marks *et al.*, 1992; DeLong *et al.*, 1993). The recombinant protein expressed from this clone catalyzes the biosynthesis of retinal from holo-CRBP with a K_m of 0.9 μM and is affected by PAO, carbenoxolone and phosphatidylcholine similarly to the partially purified and/or microsomal RoDH studied here.

Tight association between the 34 and 54 kDa bands was indicated by failure to separate the two, [^{125}I]PAO binding of 82 and 34 kDa polypeptides before and after thermal denaturation, respectively, and immunoprecipitation of RoDH activity by anti-54 kDa antiserum. Dehydrogenases generally are composed of homomultimers or heteromultimers of subunits with similar sizes and closely related amino acid sequences; SCAD in particular are composed of homomultimers. Given this and the activity of the expressed recombinant 34 kDa polypeptide, it seems that the 54 kDa band represents a distinct protein, which is not obligatory for RoDH function. The 54 kDa protein may, however, modify RoDH activity or otherwise participate in retinoid transport or metabolism. An 11-*cis*-retinol dehydrogenase purified from bovine retinal pigment epithelia also consisted of two inseparable bands on SDS−PAGE, at 33 and 66 kDa, but the enzyme activity was localized to the 33 kDa band with monoclonal antibodies (Suzuki *et al.*, 1993). This could indicate a general tendency of RoDHs to associate with "helper" proteins or proteins that have roles related to retinoid uptake and/or metabolism. On the other hand, association does not necessarily imply a functional relationship. For example, rabbit liver aldolase and fructose-1,6-bisphosphatase form a 1:1 complex (MacGregor *et al.*, 1980). Similarly, rat liver acetyl-CoA acetyltransferase and glutamate dehydrogenase copurify and coimmunoprecipitate, which indicates little more than they are nearest neighbors (Schwerdt *et al.*, 1991).

In summary, microsomal RoDH has been identified as a 34 kDa polypeptide with the characteristics of a SCAD, a family of enzymes that catalyze the metabolism of steroids and prostaglandins. The retinoid receptors belong to a superfamily of sterol/lipid hormone receptors (Evan, 1988), and the retinoid binding proteins belong to a superfamily of lipid/sterol-binding proteins (Clarke & Armstrong, 1989). Therefore, it would not be surprising teleologically if a retinoid-activating enzyme should belong to a superfamily of enzymes that catalyze lipid/sterol metabolism.

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